

(a) two variable domains, each variable domain independently an amino acid sequence comprising at least one group of three complementarity determining regions (CDRs) interposed between framework regions (FRs), which variable domains are linked a polypeptide linker to form a single polypeptide chain in which said framework and complementarity determining regions together defining a variable region binding domain which derived from a human immunoglobulin, wherein (i) is can be immunologically reactive with an said c-erbB-2 or c-erbB-2 related tumor antigen, and (ii) each group of three CDRs is selected from the group consisting of amino acid residue numbers 31-35, 50-66, 99-104, of SEQ ID NO:6, amino acid residue numbers 157-167, 183-189, 222-230 of SEQ ID NO:6, amino acid residues 31-37, 52-68, 101-110 of SEQ ID NO:2, and amino acid residue numbers 159-169, 185-191, 224-233 of SEQ ID NO:2.

(b) a third amino acid sequence, being part of said single polypeptide chain, having a biological activity independent of said immunologically reactivity.

C. Amendment of Inventorship

Please delete Lou L. Houston and David B. Ring as inventors. As described in the attached Petition Under 37 C.F.R. § 1.48(b), the invention of the amended claims is the invention of James S. Huston and Hermann Opperman.

D. Amendment of Specification

A marked up version of the specification (without claims) showing the amendments is provided as Exhibit A. The text is reproduced verbatim from the specification of US Patent 5,091,513 (which was incorporated by reference in the instant application) except for reference to Fig. 1A and Figs. 2A-2D and Fig. 3, now referred to as Figs. 7 and 8A-8D.

- Please add new Figures 7 and 8A-8D, attached as Exhibit B. These Figures correspond to Fig. 1A and Figs. 2A-2D of US Patent 5,091,513.

- At page 1, under the heading Cross Reference to Related Applications, and after the sentence "This application is a continuation-in-part of copending application Serial No. 07/831,967 filed February 6, 1992, incorporated herein by reference" Please insert the phrase:

--This application is also a continuation-in-part of U.S. Ser. 08/575,724 filed Dec. 18, 1995, now US Patent 6,207,804, which is a continuation of U.S. Ser. No. 08/139,901, filed Oct. 19, 1993, now U.S. Pat. No. 5,476,786, which is a continuation of U.S. Ser. No. 07/850,228, filed Mar. 12, 1992, now abandoned, which is a continuation of U.S. Ser. No. 07/213,671, filed Jun. 30, 1988, now U.S. Pat. No. 5,132,405, which is a continuation of U.S. Ser. No. 07/052,800, filed May 21, 1987, now abandoned. --

- At page 6, directly after the heading Summary of the Invention, please insert the following text:

A class of novel biosynthetic polypeptides has now been designed and engineered which comprise biosynthetic antibody binding sites, that is, "BABS" or chimeric polypeptides defining structure capable of selective antigen recognition and preferential antigen binding.

In its broadest aspects, this invention features polypeptides comprising biosynthetic antibody binding sites, DNA encoding these polypeptides prepared by recombinant DNA techniques, vectors comprising these DNAs, and methods for the production of these polypeptides.

In one aspect, the invention is based on the observation that three subregions of the variable domain of each of the heavy and light chains of native immunoglobulin molecules collectively are responsible for antigen recognition and binding. Each of these subregions, called herein "complementarity determining regions" or CDRs, consists of one of the hypervariable regions or loops and of selected amino acids or amino acid sequences disposed in the framework regions which flank that particular hypervariable region. It has now been discovered that framework regions from diverse species are effective to maintain CDRs from diverse other species in proper conformation so as to achieve true immunochemical binding properties in a biosynthetic protein. Thus, BABS produced in accordance with the invention comprise biosynthetically produced novel sequences of amino acids defining polypeptides designed to bind with a preselected antigenic material. The structure of these synthetic polypeptides is unlike that of naturally occurring antibodies, fragments thereof, or known synthetic polypeptides or "chimeric antibodies" in that the regions of the BABS responsible for specificity and affinity of binding, (analogous to native antibody variable regions) are themselves chimeric, e.g., comprise amino acid sequences homologous to portions of at least two different antibody molecules.

The invention thus provides a chimeric polypeptide defining a region capable of selective antigen binding and recognition. This chimeric polypeptide comprises amino acid sequences homologous to portions of the CDRs of the variable domain of one immunoglobulin light or heavy chain, and other sequences homologous to the framework regions, or FRs, of the variable domain of a second, different immunoglobulin light or heavy chain. Polypeptides so constructed bind a specific preselected antigen determined by the CDRs. Preferably, the chimeric polypeptides comprise an amino acid sequence

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homologous to at least a portion of the variable regions of a mammalian immunoglobulin, such as those of mouse, rat, or human origin. In one preferred embodiment, the biosynthetic antibody binding site comprises FRs homologous with a portion of the FRs of a human immunoglobulin and CDRs homologous with CDRs from a mouse immunoglobulin. This type of chimeric polypeptide displays the antigen binding specificity of the mouse immunoglobulin, while its human framework minimizes human immune reactions. In addition, the chimeric polypeptide may comprise other amino acid sequences. It may comprise, for example, a sequence homologous to a portion of the constant domain of an immunoglobulin, but preferably is free of constant regions (other than FRs).

The invention also provides a single chain composite polypeptide having antigen binding abilities, and comprising a pair of amino acid sequences homologous or analogous respectively to the variable regions of an immunoglobulin light and heavy chain, (linked V_H - V_L or single chain Fv). Both V_H and V_L may copy natural monoclonal sequences, or one or both of the chains may comprise a CDR-FR construct of the type described above. The separate polypeptides analogous to the variable regions of the light and heavy chains are held together by a polypeptide linker.

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This type of chimeric polypeptide is thus a single chain composite polypeptide comprising a complete antibody binding site. This single chain composite polypeptide has a structure patterned after tandem V_H and V_L domains, but with the carboxyl terminal of one attached through an amino acid sequence to the amino terminal of the other. It thus comprises an amino acid sequence which is homologous to a portion of the variable region of an immunoglobulin heavy chain (V_H) peptide bonded to a second amino acid sequence which is homologous to a portion of the variable region of an immunoglobulin light chain (V_L). The linking amino acid sequence may or may not itself be antigenic or biologically active. In addition, either the amino or carboxyl terminal ends of these chimeric, single chain Fvs may be attached to an amino acid sequence which itself is bioactive to produce a bifunctional or multifunctional protein. For example, the synthetic Fv may include a leader or trailer sequence defining a polypeptide having enzymatic activity, independent affinity for an antigen different from the antigen to which the chimeric Fv is directed, or having other functions such as to provide a convenient site of attachment for a radioactive atom, or simply to enhance expression in procaryotic host cells or yeasts.

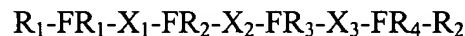
Such tandem arrangement of V_H and V_L polypeptides can increase the stability of the antigen binding site and facilitate its coupling to proteins utilized in drug targeting and moieties useful in imaging. The therapeutic use of such chimeric Fvs provide a number of advantages over larger fragments or complete antibody molecules: they are often quite stable and less immunogenic; they can penetrate body tissues more rapidly for purposes of imaging or drug delivery because of their smaller size; and they can facilitate accelerated clearance of targeted isotopes or drugs.

Other embodiments of the invention comprise multifunctional polypeptides consisting of one or more single chain Fvs either linked V_H and V_L dimers, individual V_L or V_H , or any of the foregoing comprising CDRs and FRs from different or the same immunoglobulins, linked to a second functional protein domain such as, for example, a toxin, enzyme, or site of attachment to an immobilization matrix. Yet another

embodiment is a polypeptide comprising several identical or non-identical BABS which recognize a group of antigenic determinants that are periodic or closely spaced in their normal environment, e.g., on a cell surface. This arrangement confers greatly augmented affinity and/or specifically on the BABS-containing protein analogous to, for example, the way IgM (containing 10 Fabs) binds to the surfaces of certain cells.

In other aspects, the invention provides DNA sequences encoding chimeric polypeptides of the type described above, vectors including such sequences, and methods employing the DNAs and vectors for producing the polypeptides.

A novel method of producing BABS involves the construction of a DNA containing three polynucleotide sequences (X_1 , X_2 and X_3). Each of the sequences contain restriction sites proximal its 3' and 5' ends, and each is flanked by polynucleotide sequences (FR_1 , FR_2 , FR_3 and FR_4) encoding selected framework region (FR) amino acid sequences homologous to a portion of the variable domain of an immunoglobulin. This DNA has the structure:



where R_1 is a 5' phosphate group or polynucleotide sequence and R_2 is a 3' hydroxyl group or polynucleotide sequence. The X polynucleotide sequences may be selectively excised using restriction enzymes and replaced by other DNA sequences encoding the CDR amino acid sequences of a variable domain of a selected immunoglobulin. This type of DNA sequence may encode at least part of the variable region of either or both a heavy or light chain of an immunoglobulin and may, in addition, comprise a third phosphodiester-linked nucleotide or polynucleotide sequence of a nature and function described above.

In yet another aspect, the invention provides a method for producing intact biosynthetic antibody binding sites or native Fv free of all or substantially all constant region amino acids. The method involves enzymatic digestion of chimeric immunoglobulin or at least Fab regions which have been engineered to contain preferential proteolytic cleavage sites located between the variable and constant regions of the immunoglobulin heavy and light chains. Digestion of the intact immunoglobulin with the appropriate protease yields a complete antigen binding site or Fv fragment. This approach works well in myeloma or hybridoma expression systems.

Accordingly, it is an object of this invention to provide novel proteins comprising biosynthetic antibody binding sites including an amino acid sequence homologous to specific portions of the variable region of immunoglobulin light chain and/or heavy chain, to provide DNA sequences which encode the biosynthetic antibody binding sites, and to provide replicable expression vectors capable of expressing DNA sequences encoding the biosynthetic antibody binding sites. Another object is to provide a generalized method for producing biosynthetic antibody binding site polypeptides of any desired specificity. ✕

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- At page 21, after line 9 in the section titled Brief Description of the Drawings, please insert the following text:

FIG. 7 is a schematic representation of an intact IgG antibody molecule containing two light chains, each consisting of one variable and one constant domain, and two heavy chains, each consisting of one variable and three constant domains.

FIG. 8A-8D are schematic representations of some of the classes of reagents constructed in accordance with the invention, each of which comprises a biosynthetic antibody binding site.

- At page 26, after line 26 in the section titled Detailed Description of the Invention, please insert the following text:

As is now well known, Fv, the minimum antibody fragment which contains a complete antigen recognition and binding site, consists of a dimer of one heavy and one light chain variable domain in tight, noncovalent association. It is in this configuration that the three complementarity determining regions of each variable domain interact to define an antigen binding site on the surface of the VH -VL dimer. Collectively, the six complementarity determining regions confer antigen binding specificity to the antibody. FRs flanking the CDRs have a tertiary structure which is essentially conserved in native immunoglobulins of species as diverse as human and mouse. These FRs serve to hold the CDRs in their appropriate orientation. The constant domains are not required for binding function, but may aid in stabilizing VH -VL interaction. Even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than an entire binding site (Painter et al., 1972, Biochem. 11: 1327-1337).

This knowledge of the structure of immunoglobulin proteins has now been exploited to develop biosynthetic antibody binding sites provided by this invention.

The biosynthetic antibody binding sites embodying the invention are biosynthetic in the sense that they are synthesized in a cellular host made to express a synthetic DNA, that is, a recombinant DNA made from ligation of plural, chemically synthesized oligonucleotides, or by ligation of fragments of DNA derived from the genome of a hybridoma, mature B cell clone, or a cDNA library derived from such natural sources. The proteins of the invention are properly characterized as "antibody binding sites" in that these synthetic molecules are designed specifically to have at least some affinity for a preselected antigenic substance. The polypeptides of the invention are antibody-like in that their structure is patterned after regions of native antibodies known to be responsible for antigen recognition.

More specifically, the structure of these biosynthetic proteins in the region which impart the binding properties to the protein, is analogous to the Fv region of a natural antibody. It comprises a series of regions consisting of amino acids defining at least three polypeptide segments which together form the tertiary molecular structure responsible for affinity and binding. These regions are herein called complementarity determining regions or CDRs. These CDR regions are held in appropriate conformation by polypeptide segments analogous to the framework regions of the Fv fragment of natural antibodies.

The term CDR, as used herein, refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site, or a synthetic polypeptide which mimics this function. CDRs typically are not wholly homologous to hypervariable regions of natural Fvs, but rather also include specific amino acids or amino acid sequences which flank the hypervariable region and have heretofore been considered framework not directly determinative of complementarity. The term FR, as used herein, refers to amino acid sequences interposed between CDRs.

The CDR and FR polypeptide segments are designed empirically based on sequence analysis of the Fv region of preexisting antibodies or of the DNA encoding them. In one embodiment, the amino acid sequences constituting the FR regions are analogous to the FR sequences of a first preexisting antibody, for example, a human IgG. The amino acid sequences constituting the CDR regions are analogous to the sequences from a second, different preexisting antibody, for example, the CDRs of a murine IgG. Alternatively, the CDRs and FRs from a single preexisting antibody from, e.g., an unstable or hard to culture hybridoma, may be copied in their entirety.

Practice of the invention enables the design and biosynthesis of various reagents, all of which are characterized by a region having affinity for a preselected antigenic substance. Other regions of the biosynthetic protein are designed with the particular planned utility of the protein in mind. Thus, if the reagent is designed for intravascular use in mammals, the FR regions comprise amino acids similar or identical to at least a portion of the framework region amino acids of antibodies native to that mammalian species. On the other hand, the amino acids comprising the CDRs may be analogous to a portion of the amino acids from the hypervariable region (and certain flanking amino acids) of an antibody having a known affinity and specificity, e.g., a murine or rat monoclonal antibody.

Other sections, e.g., CH and CL, of native immunoglobulin protein structure need not be present and normally are intentionally omitted from the biosynthetic proteins of this invention. However the BABS of the invention may comprise additional polypeptide regions defining a bioactive region, e.g., a toxin or enzyme, or a site onto which a toxin or a remotely detectable substance can be attached.

The clinical administration of the BABS of the invention, which display the activity of native, relatively small Fv, VH, or VL fragments, affords a number of advantages over the use of larger fragments or entire antibody molecules. The BABS of this invention offer fewer cleavage sites to circulating proteolytic enzymes and thus offer greater stability. They reach their target tissue more rapidly, and are cleared more quickly from the body. They also have reduced immunogenicity. In addition, their smaller size facilitates coupling to other molecules in drug targeting and imaging application.

The invention thus provides intact biosynthetic antibody binding sites analogous to VH-VL dimers, either non-covalently associated, disulfide bonded, or linked by a polypeptide sequence to form a composite VH-VL or VL-VH polypeptide which is essentially free of the remainder of the antibody molecule. The invention also provides proteins analogous to an independent VH or VL domain. Any of these proteins may be

provided in a form linked to amino acid sequences exclusive of those of the variable domain, for example, to amino acids analogous or homologous to proteins of a constant domain, or another bioactive molecules such as a hormone or toxin. A proteolytic cleavage site can also be designed into the region separating the variable region-like sequences from other pendant sequences so as to facilitate cleavage of intact VH and/or VL, free of other protein.

FIGS. 8A, 8B, 8C, and 8D illustrate four examples of protein structures embodying the invention that can be produced by following the teaching disclosed herein. All are characterized by one or two biosynthetic polypeptide segments defining a binding site 3, and comprising amino acid sequences comprising CDRs and FRs, often derived from different immunoglobulins, or sequences homologous to a portion of CDRs and FRs from different immunoglobulins. FIG. 8A depicts a single chain Fv comprising a polypeptide 10 having an amino acid sequence analogous to the variable region of an immunoglobulin heavy chain, bound through its carboxyl end to a polypeptide linker 12, which in turn is bound to a polypeptide 14 having an amino acid sequence analogous to the variable region of an immunoglobulin light chain. Of course, the light and heavy chain domains may be in reverse order. The linker 12 should be at least long enough (e.g., about 15 amino acids or about 40A) to permit the chains 10 and 14 to assume their proper conformation. The linker 12 may comprise an amino acid sequence homologous to a sequence identified as "self" by the species into which it will be introduced, if drug use is intended. Unstructured, hydrophilic amino acid sequences are preferred. It may also comprise a bioactive polypeptide such as a cell toxin which is to be targeted by the binding site, or a segment easily labeled by a radioactive reagent which is to be delivered, e.g., to the site of a tumor comprising an epitope recognized by the binding site. Other proteins or polypeptides may be attached to either the amino or carboxyl terminus of protein of the type illustrated in FIG. 8A. As an example, a helically coiled polypeptide structure illustrating a leader comprising a protein A fragment is shown extending from the amino terminal end of VH domain 10.

FIG. 8B illustrates two separate chains non-covalently associated and defining a binding site 3. It comprises separate peptides 16 and 18 comprising a chimeric VH and VL of the type described above. The carboxyl terminus of each protein chain may be designed to include one or more cysteine residues so that oxidation of properly folded structures produces disulfide bonds (see FIG. 8C) further stabilizing the BABS. Either or both of the polypeptides may further comprise a fused protein imparting other biological properties to the reagent in addition to the ability to bind to the antigen as specified by the interaction of the triplet CDRs on the respective polypeptides 16 and 18.

FIG. 8D depicts another type of reagent, comprising only one set of three CDRs, e.g., analogous to a heavy chain variable region, which retains a measure of affinity for the antigen. Attached to the carboxyl end of the polypeptide comprising the FR and CDR sequences constituting the binding site 3 is a Pendant Protein P consisting of, for example, a toxin, therapeutic drug, binding protein, enzyme or enzyme fragment, site of attachment for an imaging agent (e.g., to chelate a radioactive ion such as Indium), or site of attachment to an immobilization matrix so that the BABS can be used in affinity chromatography.

Of course, the protein may comprise more than one binding site or copies of a single binding site, and a number of other functional regions.

As is evidenced from the foregoing, the invention provides a large family of reagents comprising proteins, at least a portion of which defines a binding site patterned after the variable region or regions of natural immunoglobulins. It will be apparent that the nature of any protein fragments linked to the BABS, and used for reagents embodying the invention, are essentially unlimited, the essence of the invention being the provision, either alone or linked in various ways to other proteins, of binding sites having specificities to any antigen desired.

The BABS of the invention are designed at the DNA level. The chimeric or synthetic DNAs are then expressed in a suitable host system, and the expressed proteins are collected and renatured if necessary.

The ability to design the BABS of the invention depends on the ability to determine the sequence of the amino acids in the variable region of monoclonal antibodies of interest, or the DNA encoding them. Hybridoma technology enables production of cell lines secreting antibody to essentially any desired substance that produces an immune response. RNA encoding the light and heavy chains of the immunoglobulin can then be obtained from the cytoplasm of the hybridoma, and the 5' end portion of the mRNA can be used to prepare the cDNA for subsequent sequencing, or the amino acid sequence of the hypervariable and flanking framework regions can be determined by amino acid sequencing of the H and L chains and their V region fragments. Such sequence analysis is now conducted routinely. This knowledge permits one to design synthetic genes encoding FR and CDR sequences which likely will bind the antigen. These synthetic genes are then prepared using known techniques, or using the technique disclosed below, and then inserted into a suitable host, expressed, and purified. Depending on the host cell, renaturation techniques may be required to attain proper conformation. The various proteins are then tested for binding ability, and one having appropriate affinity is selected for incorporation into a reagent of the type described above. If necessary, point substitutions seeking to optimize binding may be made in the DNA using conventional cassette mutagenesis or other protein engineering methodology.

Of course, the processes for manipulating, amplifying, and recombining DNA which encode amino acid sequences of interest are generally well known in the art, and therefore, not described in detail herein. Methods of identifying and isolating genes encoding antibodies of interest are well understood, and described in the patent and other literature. In general, the methods involve selecting genetic material coding for amino acids which define the CDRs and FRs of interest according to the genetic code.

Accordingly, the construction of DNAs encoding BABS as disclosed herein can be done using known techniques involving the use of various restriction enzymes which make sequence specific cuts in DNA to produce blunt ends or cohesive ends, DNA ligases, techniques enabling enzymatic addition of sticky ends to blunt-ended DNA, construction of synthetic DNAs by assembly of short or medium length oligonucleotides, cDNA synthesis techniques, and synthetic probes for isolating immunoglobulin genes. Various promoter sequences and other regulatory DNA sequences used in achieving expression, and various types of host cells are also known and available. Conventional

transfection techniques, and equally conventional techniques for cloning and subcloning DNA are useful in the practice of this invention and known to those skilled in the art. Various types of vectors may be used such as plasmids and viruses including animal viruses and bacteriophages. The vectors may exploit various marker genes which impart to a successfully transfected cell a detectable phenotypic property that can be used to identify which of a family of clones has successfully incorporated the recombinant DNA of the vector.

One method for obtaining DNA encoding the BABS disclosed herein is by assembly of synthetic oligonucleotides produced in a conventional, automated, polynucleotide synthesizer followed by ligation with appropriate ligases. For example, overlapping, complementary DNA fragments comprising 15 bases may be synthesized semi manually using phosphoramidite chemistry, with end segments left unphosphorylated to prevent polymerization during ligation. One end of the synthetic DNA is left with a "sticky end" corresponding to the site of action of a particular restriction endonuclease, and the other end is left with an end corresponding to the site of action of another restriction endonuclease. Alternatively, this approach can be fully automated. The DNA encoding the BABS may be created by synthesizing longer single strand fragments (e.g., 50-100 nucleotides long) in, for example, a Biosearch oligonucleotide synthesizer, and then ligating the fragments.

Still another method of producing the BABS of the invention is to produce a synthetic DNA encoding a polypeptide comprising, e.g., human FRs, and intervening "dummy" CDRs, or amino acids having no function except to define suitably situated unique restriction sites. This synthetic DNA is then altered by DNA replacement, in which restriction and ligation is employed to insert synthetic oligonucleotides encoding CDRs defining a desired binding specificity in the proper location between the FRs.

This technique is dependent upon the ability to cleave a DNA corresponding in structure to a variable domain gene at specific sites flanking nucleotide sequences encoding CDRs. These restriction sites in some cases may be found in the native gene. Alternatively, non-native restriction sites may be engineered into the nucleotide sequence resulting in a synthetic gene with a different sequence of nucleotides than the native gene, but encoding the same variable region amino acids because of the degeneracy of the genetic code. The fragments resulting from endonuclease digestion, and comprising FR-encoding sequences, are then ligated to non-native CDR-encoding sequences to produce a synthetic variable domain gene with altered antigen binding specificity. Additional nucleotide sequences encoding, for example, constant region amino acids or a bioactive molecule may also be linked to the gene sequences to produce a bifunctional protein.

The expression of these synthetic DNAs can be achieved in both prokaryotic and eucaryotic systems via transfection with the appropriate vector. In *E. coli* and other microbial hosts, the synthetic genes can be expressed as fusion protein. Expression in eucaryotes can be accomplished by the transfection of DNA sequences encoding CDR and FR region amino acids into a myeloma or other type of cell line. By this strategy intact hybrid antibody molecules having hybrid Fv regions and various bioactive proteins including a biosynthetic binding domain may be produced. For fusion protein expressed in bacteria subsequent proteolytic cleavage of the isolated VH and VL fusions can be

performed to yield free VH and VL, which can be renatured, and reassociated (or used separately) to obtain an intact biosynthetic, hybrid antibody binding site.

Heretofore, it has not been possible to cleave the heavy and light chain region to separate the variable and constant regions of an immunoglobulin so as to produce intact Fv, except in specific cases not of general utility. However, one method of producing BABS in accordance with this invention is to redesign an immunoglobulin at the DNA level so as to alter its specificity and so as to incorporate a cleavage site and "hinge region" between the variable and constant regions of both the heavy and light chains. Such chimeric antibodies can be produced in transfectomas or the like and subsequently cleaved using a preselected endopeptidase. The engineering principles involved in these easily cleaved constructs are disclosed in detail in copending U.S. application Ser. No. 028,484 filed Mar. 20, 1987 by Huston et al.

The hinge region is a sequence of amino acids which serve to promote efficient cleavage by a preselected cleavage agent at a preselected, built-in cleavage site. It is designed to promote cleavage preferentially at the cleavage site when the polypeptide is treated with the cleavage agent in an appropriate environment.

The hinge can take many different forms. Its design involves selection of amino acid residues (and a DNA fragment encoding them) which impart to the region of the fused protein about the cleavage site an appropriate polarity, charge distribution, and stereochemistry which, in the aqueous environment where the cleavage takes place, efficiently exposes the cleavage site to the cleavage agent in preference to other potential cleavage sites that may be present in the polypeptide, and/or to improve the kinetics of the cleavage reaction. In specific cases the amino acids of the hinge are selected and assembled in sequence based on their known properties, and then the fused polypeptide sequence is expressed, tested, and altered for empirical refinement.

The hinge region is free of cysteine. This enables the cleavage reaction to be conducted under conditions in which the protein assumes its tertiary conformation, and may be held in this conformation by intramolecular disulfide bonds. It has been discovered that in these conditions access of the protease to potential cleavage sites which may be present within the target protein is hindered. The hinge region may comprise an amino acid sequence which includes one or more proline residues. This allows formation of a substantially unfolded molecular segment. Aspartic acid, glutamic acid, arginine, lysine, serine, and threonine residues maximize ionic interactions and may be present in amounts and/or in sequence which renders the moiety comprising the hinge water soluble.

In the case of single chain Fv comprising fused H and L chains, the cleavage site preferably is immediately adjacent the Fv polypeptide and comprises one or a sequence of amino acids exclusive of any one or sequence found in the amino acid structure of the BABS. Where BABS VH and VL regions are on separate chains (i.e., see FIG. 7), the cleavage sites may be either immediately adjacent their C-terminal ends, thereby releasing Fv dimer of VH and VL upon appropriate cleavage (i.e., to yield the species of FIG. 8B), or may follow pendant polypeptides with or without cysteine that yield, respectively, the species of FIG. 8C or 8D upon digestion.

The cleavage site preferably is designed for cleavage by a specific selected agent. Endopeptidases are preferred, although non-enzymatic (chemical) cleavage agents may be used. Many useful cleavage agents, for instance, cyanogen bromide, dilute acid, trypsin, *Staphylococcus aureus* V-8 protease, post proline cleaving enzyme, blood coagulation Factor Xa, enterokinase, and renin, recognize and preferentially or exclusively cleave particular cleavage sites. One currently preferred cleavage agent is V-8 protease. The currently preferred cleavage site is a Glu residue. Other useful enzymes recognize multiple residues as a cleavage site, e.g., factor Xa (Ile-Glu-Gly-Arg) or enterokinase (AsP-Asp-AsP-Asp-Lys).

With the help of a computer program and known variable region DNA sequences, synthetic VL and VH genes may be designed which encode native or near native FR and CDR amino acid sequences from an antibody molecule, each separated by unique restriction sites located as close to FR-CDR and CDR-FR borders as possible. Alternatively, genes may be designed which encode native FR sequences which are similar or identical to the FRs of an antibody molecule from a selected species, each separated by "dummy" CDR sequences containing strategically located restriction sites. These DNAs serve as starting materials for producing BABS, as the native or "dummy" CDR sequences may be excised and replaced with sequences encoding the CDR amino acids defining a selected binding site. Alternatively, one may design and directly synthesize native or near-native FR sequences from a first antibody molecule, and CDR sequences from a second antibody molecule. Any one of the VH and VL sequences described above may be linked together directly, either via an amino acids chain or linker connecting the C-terminus of one chain with the N-terminus of the other, or via C-terminal cysteine residues on each of the VH and VL.

These genes, once synthesized, may be cloned with or without additional DNA sequences coding for, e.g., an antibody constant region, or a leader peptide which facilitates secretion or intracellular stability of a fusion polypeptide. The genes then can be expressed directly in an appropriate host cell, or can be further engineered before expression by the exchange of FR, CDR, or "dummy" CDR sequences with new sequences. This manipulation is facilitated by the presence of the restriction sites which have been engineered into the gene at the FR-CDR and CDR-FR borders.

The engineered genes can be expressed in appropriate prokaryotic hosts such as various strains of *E. coli*, and in eucaryotic hosts such as Chinese hamster ovary cell, mouse myeloma, and human myeloma/transfectoma cells.

For example, if the gene is to be expressed in *E. coli*, it may first be cloned into an expression vector. This is accomplished by positioning the engineered gene downstream from a promoter sequence such as Trp or Tac, and a gene coding for a leader peptide such as fragment B of protein A (FB). The resulting expressed fusion protein accumulates in refractile bodies in the cytoplasm of the cells, and may be harvested after disruption of the cells by French press or sonication. The refractile bodies are solubilized, and the expressed proteins refolded and cleaved by the methods already established for many other recombinant proteins.

If the engineered gene is to be expressed in myeloma cells, the conventional expression system for immunoglobulins, it is first inserted into an expression vector

containing, for example, the Ig promoter, a secretion signal, immunoglobulin enhancers, and various introns. This plasmid may also contain sequences encoding all or part of a constant region, enabling an entire part of a heavy or light chain to be expressed. The gene is transfected into myeloma cells via established electroporation or protoplast fusion methods. Cells so transfected can express V_L or V_H fragments, V_L - V_H heterodimers, V_H - V_L or V_L - V_H single chain polypeptides, complete heavy or light immunoglobulin chains, or portions thereof, each of which may be attached in the various ways discussed above to a protein domain having another function (e.g., cytotoxicity).

Vectors containing a heavy chain V region (or V and C regions) can be cotransfected with analogous vectors carrying a light chain V region (or V and C regions), allowing for the expression of noncovalently associated Fvs (or complete antibody molecules).

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